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THE MANIPULATION AND IDENTIFICATION OF THE FREE-SWIMMING MASTIGOPHORA OF FRESH WATERS

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Not the least among the problems that confront the worker in systematic protozoology is the identification of the minute, free-swimming Mastigophora, representatives of which occur in all cultures, often in great numbers both of individuals and of larger groups. The writer's attempts to find some means of making their identification easier and more certain have resulted in the working out of several methods of dealing with these elusive forms, methods which have been used with success in the laboratory examination of material. They are here presented with the hope that they may be a similar assistance to others.

For the purposes of identification it has been found helpful arbitrarily to divide the Mastigophora into two great groups, on the basis of size; placing those the majority of whose species measure about 12 microns or less along the antero-posterior axis in one group, and those of greater magnitude in the other. It is among the species in the first group that the difficulties in identification seem usually to occur, and it is with this division, therefore, that this paper deals.

The genera included within this first arbitrary division fall within the first four orders of the mastigophora, thus:

SUBPHYLUM MASTIGOPHORA

Order Monadida
    Genera: *Mastigamoeba*
        Cercobodo
        Cercomonas
        Physomonas

ORDER HETEROMASTIGIDA
Genera: *Elvirea*
Dinomonas
Pleuromonas
Spiromonas

ORDER PHYTOMASTIGIDA
Genera: *Amphimonas*
Hexamita

ORDER EUGLENIDA
Genera: *Cryptoglena*
Notosolenus

Almost all the mastigophora, but more especially the minute forms, vary in size and shape within the limits of the species. Individuals of apparently the same species are often found half, and even a fourth, of the size which is normal for that species, while abnormally large forms are more rarely encountered. It is the smaller forms which are the most puzzling. These are probably due to: (1) the division of the adults, for binary fission along the line of the antero-posterior axis is the prevailing mode of reproduction among the Mastigophora. Where division is occurring rapidly, in a crowded culture many such forms will be found. Often the young again subdivide, even before reaching adult proportions. This gives rise to individuals of a species varying widely in size, (2) to the fact that the adults themselves vary in size, even where division is not rapidly taking place, and (3) to the possible production in some forms of swarm spores. Possibly when the life histories of a greater number of the smaller flagellates become known, it will be found that many species multiply not only by binary fission, but by multiple fission as well, either within or without the cyst. It is not unusual to find individuals of a colonial form which have freed themselves, or have been broken away, from the parent community, and which are, temporarily, at least, living an independent existence. Adventitious individuals may be derived from such colonial mastigophora as *Spongomonas*, *Anthophysa*, *Dendromonas*, *Uroglena*, and *Ramosonema*.

The following key, which was devised by the writer, has been found helpful in the identification of the minute forms, and in fixing in mind the characters that are the most prominent, and that are useful, under the microscope, in identification. The key includes practically all the forms of the minute mastigophora that are likely to be found in the waters of bogs, ponds, roadside ditches, creeks, and brooks, and putrefying infusions, in so far as these have been accorded systematic place. These forms occur over and over again, and their identification has been found to be a matter of less difficulty than was at first thought, before a definite plan for handling them was formulated.

KEY TO THE MINUTE MASTIGOPHORA, THOSE ROUGHLY ABOUT OR BELOW 12 MICRONS

A. Antero-posterior diameter normally about, or less than 8 microns.
  B. Flagella 2, body kidney-shaped, the smallest of the mastigophora .... *Pleuromonas*
  BB. Flagella 3, two trailing, one extended forward..... *Elvirea*
AA. Antero-posterior diameter normally greater than 8 microns.
  B. With one flagellum.
    C. With greenish chromatophores .... *Cryptoglena*
    CC. Without chromatophores.
      D. Flagellum stiff except at tip .... *Notosolenus*
      DD. Flagellum not stiff, pseudopodia present .... *Mastigamoeba*
  BB. With more than one flagellum.
    C. With 2 flagella.
      D. With one flagellum at each extremity of the body .... *Cercomonas*
      DD. Both flagella at same extremity of body.
        E. Body ovate, the anterior extremity the smaller .... *Dinomonas*
        EE. Body not normally ovate.
          F. Body spherical .... *Amphimonas*
          FF. Body not spherical.
            G. Body ribbon-like, twisted .... *Spiromonas*
            GG. Body not twisted.

H. Body drawn out bluntly posteriorly, obliquely truncated anteriorly .... *Physomonas*
HH. Body drawn out into an acuminate tip posteriorly, not truncated anteriorly .... *Cercobodo*
CC. Flagella more than 2.
D. Flagella 3, two trailing, one extended forward ..*Elvirea*[1]
DD. Flagella four, anteriorly extended; posterior produced into 2 filamentous appendages....*Hexamita*

In dealing with these minute, free-swimming forms, it was of first necessity to devise some means of keeping them quiet and within the field of the microscope when the higher powers were in use. Several methods both of retarding movement and of killing were used, but those which gave the best results were the following.

For the first examination of any sample, a small drop of the culture was taken and mixed on a slide with a drop of very viscous gelatine solution,[2] and the whole thoroughly stirred together. Or often several drops of the culture were mixed with an equal part of the gelatine solution in a watch crystal and used on the slide when needed. Such a preparation would not keep the protozoa confined within it alive for more than half an hour, however, due to the occlusion of the necessary oxygen.

The drop on the slide was now carefully flattened out and examined without a cover glass under a low power (16 mm. objective and 10x eyepiece) to ascertain whether the solution were of a viscosity great enough to check sufficiently the movements of the flagellates. If not, it was allowed to concentrate still more by evaporation until properly viscous, and then covered with a cover glass. Magnification with the 4 mm. objective and the 8x and 10x eyepieces was found to be great enough for the identification of most of the forms.

The gelatine used in this method must be of the best grade and perfectly fresh and clear. It may be slightly agitated before adding to the culture drop. This includes numerous minute air bubbles, around which the animals may gather and so become concentrated, if the solution is at first thin enough to allow them to make their way through it. It was found that gelatine that had stood ready made up for some time in a warm room became cloudy in appearance and stringy in texture, due to the growth of mould plants and colonies of bacteria.

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[1] *Elvirea*, because of its variability, is placed both here and in division *AA* of the key.

[2] See formulary of reagents at end of paper.

Another method of retarding the motions of the flagellates, which was partially successful with such minute forms as *Pleuromonas jaculans* and *Elvirea cionae,* was to chill the slide and its water drop thoroughly on a block of ice. This was tried in midsummer, when the sudden reduction in temperature of water that had been quite warm (the culture having stood in the sun) apparently paralyzed the organisms, but they regained their wonted activity after a few minutes' time, since the slide could not very conveniently be kept chilled under the microscope.

The favorite method of the writer for quieting without killing was to utilize a minute aquarium, of a sort that might be used even under the high powers. This was constructed by cutting out a circle of very thin typewriter manifolding paper of good grade and firm texture of slightly less diameter than that of the cover glass, and then cutting from the center of this a concentric aperture about 5 mm. in diameter. This was affixed to the slide with a ring of thin balsam or castor oil, applied with a fine camel's hair brush; the water drop placed in the center, and the cover glass, also ringed with thin balsam or oil, carefully lowered thereon. The oil or balsam sealed the cover glass, and the paper kept it from descending far enough to crush the incarcerated organisms.

At first the flagellates in such an aquarium swim about at their normal rates of speed, but after a time they become stupefied, probably because of the gradual exhaustion of the oxygen supply, and their movements become progressively slower, until finally they cease altogether.

The flagella continue to beat for some little time after the animal has come to rest.

This offers a good opportunity for the observation of the natatory habits, and, as the animals quiet down, for making a closer examination for identification; using higher powers. The smaller the water volume in such a micro-aquarium, the sooner the stupefaction of the confined organisms takes place. The forms studied in the micro-aquarium were usually stained *intra vitam* before their incarceration.

The stains most frequently used for *intra vitam* work were methylene blue (not methyl blue) and methyl green.[3] With each of these a saturated aqueous solution was prepared and diluted to the desired strength.

The stains which were used for killed specimens were methylene blue (not methyl blue), methyl green, gentian violet, and safranin.[4]

The examination of the imprisoned *intra vitam* stained animals has the advantage over the killed and stained ones that it shows the position and action of the flagella, and so leads to a more correct idea of how this should be represented in a drawing. Occasionally one meets with the drawing of a form in which the flagellum is represented as thrown into graceful undulations, whereas, in life, it may be only the tip of that organ that is motile. It was found that frequently the killing reagents caused the flagella to assume unnatural attitudes.

In both the killed and the *intra vitam*-stained animals the flagella takes the stain least of all, often appearing but very faintly, if at all, colored. In *intra vitam* staining, care must be taken not to kill the creatures with too much stain in the attempt to make the flagella stain more deeply.

Killing, in order that examination might be made with the 1.8 mm. objective, using oil, was accomplished by the use of the various well known reagents, such as tannic acid, osmic acid, acetic acid, formaldehyde, and mercuric chloride solutions.[4] The best results, however, were obtained by the use of a 1 per cent. aqueous solution of copper sulphate, a reagent which was hit upon in the attempt to find a medium in which death occurred with a minimum of distortion of the flagella. In nearly all cases the animals very gradually subsided into immobility without any distortion whatever. A .5 per cent. solution kills them much more gradually. These solutions must be made up with accuracy, and may be most delicately prepared by counting drops of water and of concentrated copper sulphate solution, as they come from the tip of a finely drawn out pipette. One hundred drops of water from a pipette the diameter of whose tip measures about 2 mm. makes a sufficient quantity to last for months.

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[3] See formulary of reagents at end of paper.

[4] See formulary of reagents at end of paper.

The killing and staining was accomplished in either of two ways, either by killing first and staining afterwards, in which case any of the killing reagents given at the end of the paper was used, followed by the stain, or by performing both operations simultaneously. This may be done by using strong stains. The material can be stained either on a slide, or in larger quantities in a watch crystal. Where the animals were extremely abundant, as they usually were in surface scums or decaying cultures, the latter method was found to be the best, for with the larger quantity of water both the killing reagent and the stain could be more delicately controlled. Several watch crystals were placed side by side and various gradations of color secured.

The killed specimens were examined at once, and fresh preparations frequently made. Complete disintegration of these tiny forms takes place a short time after they have been killed. This is preceded by a distortion of the body.

On the whole, *intra vitam* staining, with the animals retarded in the gelatine solution, or stupefied in the micro-aquarium, gave the best results. With animals treated thus, the magnifications afforded by the 8x and

10x eyepieces and the 4 mm. objective were found great enough for the majority of the forms.

In the following descriptions of the genera and species the attempt has been made to indicate the characters which are the most prominent ones of the members of each group, those by which the identification can usually be made. Hitherto less attention than the subject seems to deserve has been given to the manner in which these lower forms make their way through the water—to what may be called their natatory habits. Many forms show natatory habits which seem to be of a constancy and a distinctness sufficient to warrant their use as characteristics for identification. This feature has been given attention in the following descriptions because it has been found a helpful one in identifying the forms.

It is here suggested that the vibratory motions which some of the smaller flagellates, like *Pleuromonas* and *Elvirea,* exhibit, may be due in part to the influence of pedesis, or Brownian movements. Carpenter states[5] that all particles suspended in water below $\frac{1}{500}$ of an inch (49 microns) exhibits this phenomenon, and it is a matter of observation that the smaller the particle the more pronounced the vibration.

ORDER MONADIDA

GENUS MASTIGAMOEBA Schultze.

Body amœboid, changing shape slowly as pseudopodia are protruded, usually from the sides or posterior portion of the body. Flagellum long, fine, and not easily seen at first in its entirety, because of its rapid motion. These forms move with either a true amœboid motion, or swim by means of the flagellum. Conn states that in swimming the pseudopodia are retracted, yet we have noticed frequent exceptions in both species. The swimming of these species is clumsy, due perhaps to the irregularity of the body.

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[5] "The Microscope and Its Revelations."

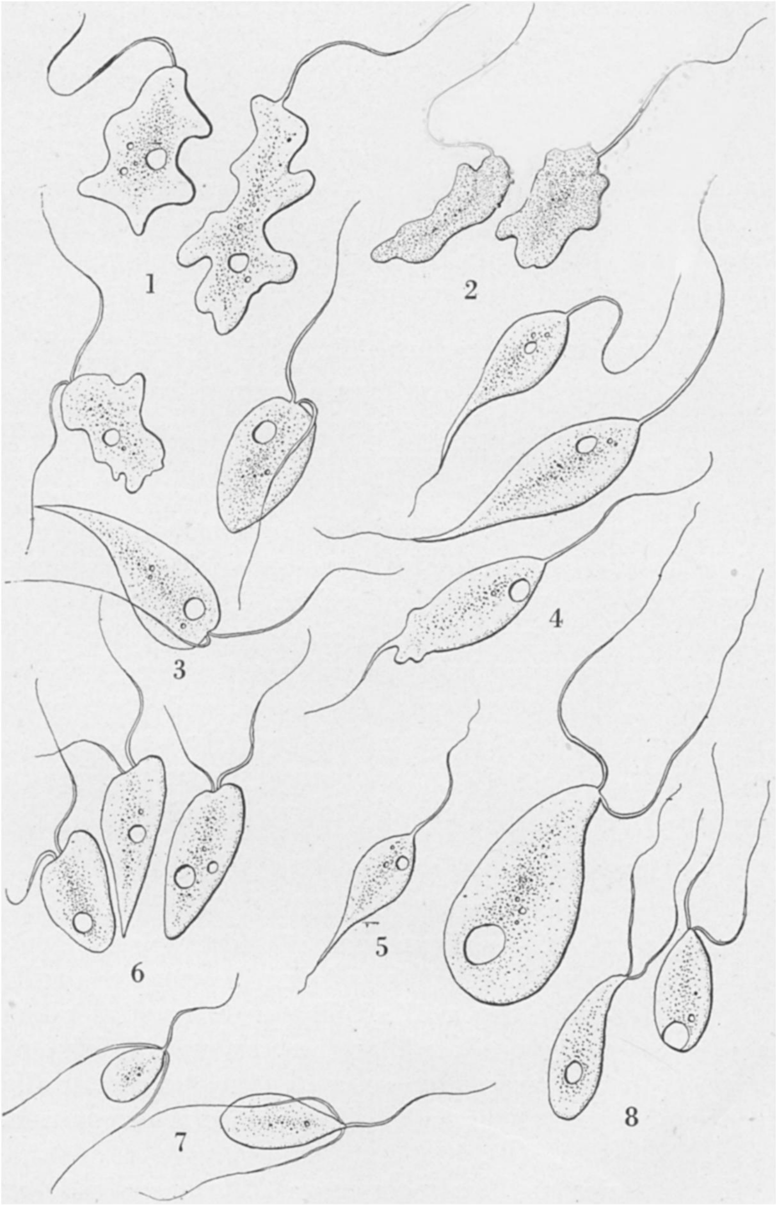

FIG. 1. *Mastigamoeba longifilum* Stokes (10–15 μ), 2 individuals.

FIG. 2. *Mastigamoeba repetans* Stokes (8–10 μ), 2 individuals.

FIG. 3. *Cercobodo mutabilis* Kent (8–12 μ), 3 individuals, 1 in the pseudopodia-bearing stage.

FIG. 4. *Cercomonas longicauda* Dujardin (10–13 μ), 3 individuals, 1 protruding pseudopodia at the posterior extremity of the body.

FIG. 5. *Cercomonas crassicauda* Dujardin (6–9 μ), 1 individual of typical form.

FIG. 6. *Physomonas elongata* Stokes (10–13 μ), 3 individuals.

FIG. 7. *Elvirea cionae* Parona (5–8 μ), 2 individuals.

FIG. 8. *Dinomonas vorax* Kent (8–15 μ), 3 individuals.

M. longifilum (Fig. 1) seems to be the most common species in the waters about Ithaca, N. Y., and New Haven, Conn., though *M. repetans* (Fig. 2) was often seen. The former is the larger, the more hyaline, and furthermore possesses at least one quite prominent contractile vacuole. No difference in the length of the flagellum in the two species could be observed.

Genus Cercobodo Kraasilstschik.

Body changeable in form from almost globular to elongate, with the posterior extremity usually more or less drawn out, frequently acuminate. This latter form is the one under which the species most frequently appears. The two flagella arise from the anterior end of the body. Often an amœboid form of body is assumed, and locomotion is effected by blunt pseudopodia.

Because of its instability of form, the one species which is the most common has been relegated successively to the genera *Dimastigamœba, Dimorpha,* and *Cercobodo.* The single contractile vacuole is usually present and prominent. The one species observed seems to conform to the *Cercobodo mutabilis* of Stokes (Fig. 3).

Genus Cercomonas Dujardin.

Body globular to ovate, pointed at the anterior and posterior extremities, from each of which arises a long flagellum, the posterior of these being the stouter, a trifle the shorter and less motile. The pseudopodia, which are occasionally produced, are not as well defined as those in the two preceding genera, and are limited to the posterior fourth of the body. These were not frequently seen.

Two species are fairly common: *C. longicauda* (Fig. 4), and *C. crassicauda* (Fig. 5). They may be distinguished by their difference in size.

Genus Physomonas Kent.

Body changeable in form, though not possessing pseudopodia, and varying from elongately ovoid to ovoid pointed at the posterior extremity. The anterior portion of the body is normally obliquely truncated, from which arise two flagella of unequal length.

P. elongata (Fig. 6) is fairly common in all waters of ponds and bogs, particularly among sphagnum, though it seems never to occur in crowded cultures.

ORDER HETEROMASTIGIDA

GENUS ELVIREA Parona.

The body is pear-shaped, and though it may elongate and contract slightly during swimming, it is quite stable in form. Of the three flagella which arise from the anterior extremity, the stouter, shorter one vibrates forward, and is the organ of locomotion. The other two trail behind.

E. cionæ (Fig. 7) is apparently not a very common species. It was found sparsely in the clear cold waters of springs and creeks.

GENUS DINOMONAS Kent.

Members of this genus resemble those of the preceding one in the general shape of the body, but they are larger and possess but two flagella, both of which extend forward from the more acute anterior extremity. The contractile vacuole is usually clearly visible and located in the rounded posterior region.

Fig. 8 was found abundantly in the scums of various infusions of grasses and leaves, and conforms to the *D. vorax* of Kent. *D. tuberculata* (Fig. 9) was often found in the same infusions with *D. vorax.* It is possible that this may be merely another form of the latter species.

GENUS PLEUROMONAS Perty.

Body either kidney-shaped or ovate, the two long flagella arising from a depression in the venter which is not, however, invariably present.

P. jaculans (Fig. 10) is often very common in stagnant waters wherever there is decomposing vegetable matter present. Some infusions will be colored a milky hue from the multitudes of these forms within them. The individuals of the species vary considerably in size, some being no greater than 2 microns along the anterio-posterior axis. The majority of the individuals, however, lie within the limits of 3 to 8 microns. They may be recognized at once by their peculiar agitation and habit of leaping or jerking about from place to place. Individuals may sometimes be discovered lying quiet, except for a gentle vibration—a motion which, it has been suggested earlier in this paper, may be due to the influence of pedesis. These quiet forms are to be found usually near the edges of masses of disintegrating material, where they are likely to be overlooked. The bodies are often particularly hyaline, and the flagella difficult to make out. Iodine (an alcoholic solution, with potassium iodide, as given under No. 2, Section B of the Formulary of Reagents at the end of the paper) as a stain after killing was found to be the best for these minute forms. In some cases the flagella could be made to take the stain well, in others not. Those killed with the stain itself seemed to be better colored than those stained after having been killed with some other reagent.

GENUS SPIROMONAS Perty.

Body leaf or ribbon-like, either flattened or more commonly twisted spirally, with one or two turns, very variable. Kent says that these forms may even assume an amœboid form of body. Of the two flagella that arise from the anterior tip of the body, one trails, one extends forward and vibrates with great rapidity.

The variable *S. angustata* (Fig. 11) seems to be quite common in infusions of all kinds, but particularly abundant in those of hay, swamp grasses, and the like.

ORDER PHYTOMASTIGIDA

GENUS AMPHIMONAS Dujardin.

Body globular, either attached by a fine posterior pedicel, or free-swimming—the latter being apparently the

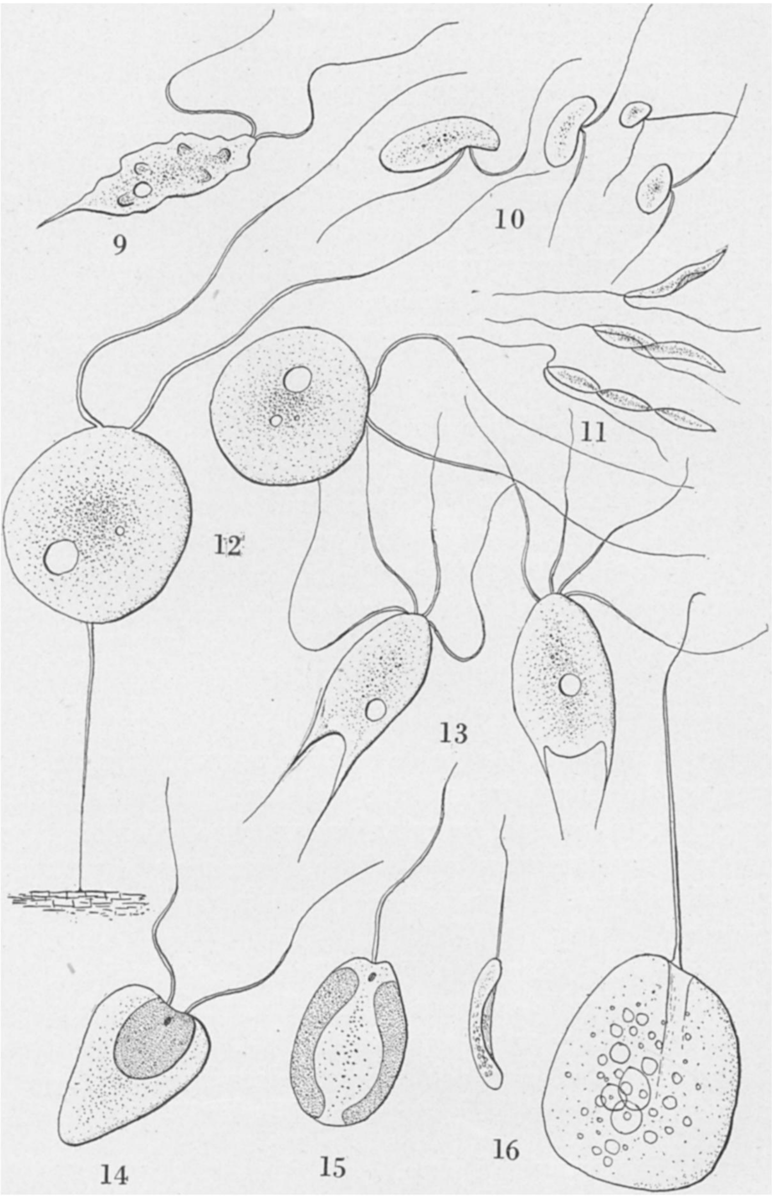

FIG. 9. *Dinomonas tuberculata* Kent (8–15 μ), 1 individual.

FIG. 10. *Pleuromonas jaculans* Perty (3–9 μ), 4 individuals.

FIG. 11. *Spiromonas angustata* Dujardin (8–10 μ), 3 individuals.

FIG. 12. *Amphimonas globosa* Kent (10–12 μ), 2 individuals, 1 free, 1 attached by a posterior pedicel.

FIG. 13. *Hexamita inflata* Dujardin (10–12 μ), 2 individuals.

FIG. 14. *Cryptoglena conica* Ehrenberg (10–12 μ), 1 individual.

FIG. 15. *Cryptoglena pigra* Ehrenberg (10–12 μ), 1 individual.

FIG. 16. *Notosolenus orbicularis* Stokes (7(?)–15 μ), 2 individuals, 1 turned sidewise to show the concavo-convex shape of the body. This latter form may be, perhaps, another species.

more common condition—by means of two long, fine, equal, anterior, rapidly vibrating flagella. In the free-swimming stage the posterior pedicel is usually absent, though occasionally individuals may be seen trailing after them pedicels of different lengths. These may be pedicels in various stages of retraction into the body.

A. globosa (Fig. 12) seems to be of rather rare occurrence in the waters of ponds and ditches among decaying aquatics.

GENUS HEXAMITA Dujardin.

Body very changeable in form, with two long, filamentous appendages arising from near the posterior extremity of the body. Flagella four, anterior, active.

H. inflata (Fig. 13) was found in water containing *Ceratophyllum* just beginning to decay.

ORDER EUGLENIDA

GENUS CRYPTOGLENA Ehrenberg.

Body oval, not changeable in form, nor varying greatly among the members of the species; possessing one greenish yellow, or greenish brown chromatophore, or two. Flagellum stout at its base, there may be present a red stigma. Swims rapidly, with an undulatory course.

Two species, *C. conica* (Fig. 14) and *C. pigra* (Fig. 15), seem to be quite common in pond waters among such smaller aquatics as *Lemna, Elodea,* etc. They are often found in water in which there is decaying vegetation, also, associated with *Euglena* and *Phacus*.

GENUS NOTOSOLENUS Stokes.

Body hollowed, resembling the bowl of a blunt, thick, wooden spoon; hyaline, colorless, and filled with a large number of large, globular, glassy granules or vacuoles. We have observed but very few individuals which did not contain these. Flagellum long, stiff, and stout, and rigid except that its distal fourth only is motile. This peculiar characteristic affords a good identification character. During swimming it often turns over and over in the water, at which times the concavo-convex shape of the body becomes appreciable.

Either individuals of the species *N. orbicularis* (Fig. 16) vary remarkably in size, or there is more than one species, differing, apparently, only in this particular. The form of the body is, however, constant.

These forms are found, unlike most of the flagellata, most abundantly in fresh waters, particularly in the clear cold waters of springs, that support little plant growth, and they occur usually near the bottom, among the sands and pebbles.

Their progression through the water is often slow and deliberate, and at such times it seems as though the tip of the flagellum were functioning as an exploratory antenna.

FORMULARY OF REAGENTS USED FOR RETARDING, KILLING ANG STAINING

1. Retarding Solutions:
   (1) Gelatine Solution.

Water	5 oz.
Gelatine	¼ oz.

Heat, to dissolve the gelatine; then allow to cool to the desired viscosity.

   (2) A 1 per cent. aq. sol. of chloretone narcotizes the animals, and gave good results with certain forms, though its manipulation was a trifle difficult.

2. Killing Reagents:
   (1) 25 per cent. aq. sol. tannic acid.
   (2) 5 per cent. aq. sol. acetic acid.
   (3) 10 per cent. aq. sol. mercuric chloride.
   (4) 1 per cent. aq. sol. formalin.
   (5) Invert slide with its suspended drop over the neck of a bottle containing a 2 per cent. sol. of osmic acid. The fumes kill the animals at once.
   (6) 1 per cent. aq. sol. copper sulphate. This gave the best results of any of the killing reagents.

3. Stains:
   A. For *intra vitam* staining. (Make up a quantity of the stain as directed, and then dilute with water to obtain desired depth of color.)

(1) Sat. aq. sol. methylene blue.
(2) Sat. aq. sol. methyl green.
(3) Sat. aq. sol. gentian violet.
(4) Sat. aq. sol. safranin.

B. For staining after killing, or for killing with the stain itself. (Make up a quantity of the stain, and dilute with water for desired color.)

(1) Sat. alcoholic sol. methylene blue.
(2) Sat. alcoholic sol. methyl green.
(3) Sat. alcoholic sol. gentian violet.
(4) Sat. alcoholic sol. safranin.
(5) Sat. alcoholic sol. iodine, with 3 per cent. potassium iodide. This gives excellent results. It is a very powerful stain, and must be used in weak solutions.